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**Prognostic and predictive role of topoisomerase alpha 2 and thymidylate synthase expression in tumor tissues of patients with adrenocortical carcinoma.**

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## **Abstract**

Topoisomerase II alpha (TOP2A) and Thymidylate Synthase (TS) are known prognostic parameters in several tumors. They are also predictors of efficacy of anthracyclines and topoisomerase inhibitors and fluoropyrimidines, respectively.

The prognostic and predictive role of tumor tissue mRNA expression of TOP2A and TS was assessed in 98 patients with adrenocortical carcinoma (ACC). Ninety-two were radically resected for stage II-III disease and 38 of them received adjuvant mitotane after surgery. Twenty-six patients with metastatic disease at first diagnosis or at the time of recurrence after surgery received the EDP-M (Etoposide, Doxorubicin=Adriamycin, Cisplatin plus mitotane). TOP2A and TS expression in ACC tissue was directly correlated. Both markers were not associated with either disease free survival (DFS) after surgery or overall survival (OS) in multivariate analyses and failed to be associated to mitotane efficacy in terms of DFS. A clinical benefit (disease response or disease stabilization) to EDP-M treatment was observed in 12/17 (71%) and 1/9 (11%) patients with high and low TOP2A expressing tumors ( $p=0.0039$ ) and 9/13 (69%) and 4/13 (31%) patients with high and low TS expressing ACC, respectively ( $p=0.049$ ). High TOP2A expression was significantly associated with longer time to progression after EDP-M.

TOP2A and TS were neither prognostic nor predictive of mitotane efficacy in ACC patients.

The predictive role of TOP2A expression of EDP-M activity suggests a significant contribution of adriamycin and etoposide for the efficacy of the EDP scheme.

## Introduction

Adrenocortical carcinoma (ACC) is a rare and aggressive malignant tumor [1,2]. Surgery is the mainstay of therapy. Although complete surgical removal of ACC is the only potentially curative approach, most of the radically resected patients are destined to relapse, often with metastases [3,4]. Mitotane is the reference systemic therapy for ACC [5]. On the basis of the results of a large retrospective multicentric study carried out at several referral centers in Italy and Germany [6], the drug is recommended to be administered in adjuvant setting in radically resected ACC with high risk of recurrence [7,8]. The recent finding that mitotane serum levels are prognostic in patients receiving the drug as adjuvant therapy provides further evidence in favor of the efficacy of this drug in this setting [9]. However, few prognostic factors are currently available to identify the patient risk of relapse and death of disease. Currently only disease stage, completeness of initial resection, and proliferation index are widely accepted prognostic factors [8,10, 11]. In the patients with metastatic disease at diagnosis or showing distant recurrence after surgery, chemotherapy plus mitotane is the best treatment strategy. The results of a multinational prospective randomized clinical study have established the combination chemotherapy with the topoisomerase II inhibitor Etoposide, the anthracycline Doxorubicin, Cisplatin plus mitotane (EDP-M) as the reference regimen for this rare disease [12]. Adjuvant mitotane therapy and EDP-M however, are toxic and their overall efficacies limited. Therefore, the establishment of predictive factors that identify a subset of patients, in whom a certain treatment would be more effective, would be highly desirable. We have previously evaluated the expression of ribonucleotide reductase large subunit 1 (RRM1) and ERCC1 genes in a multicenter (German and Italian) cohort of ACC patients and provided the first evidence *in vitro* and *in vivo* that RRM1 gene expression levels are functionally associated to mitotane sensitivity and predict response to mitotane treatment in adjuvant setting [13]. In the same series we assessed the expression of two other biomarkers with potential prognostic impact: the topoisomerase II alpha (TOP2A), an enzyme responsible for transcription, replication and chromosome condensation and segregation during cell division [14]; and the thymidylate

synthase (TS), an enzyme involved in nucleotide metabolism [15]. TOP2A is also a well known predictor of efficacy of anthracyclines and topoisomerase inhibitors [16] and TS is predictive of the efficacy of fluoropyrimidines [17]. In the present study, we explored the prognostic significance of these markers in ACC as well as their predictive role for efficacy of adjuvant mitotane and of EDP-M in patients with advanced disease.

## **Patients and Methods**

### *Patients.*

Ninety-eight patients with radically resected ACC between 1989 and 2007 at the San Luigi Hospital of Orbassano, University of Turin, Italy (51 patients) and 35 centers in Germany coordinated by the German ACC Registry (47 patients) were included according to the following eligibility criteria: 1) age of 18 years or older; 2) histologically confirmed diagnosis of ACC after central pathologic revision (MV, MP); 3) complete resection of primary ACC, 4) availability of follow-up information, 5) availability of representative paraffin-embedded tissue block(s). All patients fulfilling these criteria were included in the study. Ninety-two patients were already considered in the previously published series that have tested ERCC1 and RRM1 [13]. In the present paper we also considered six patients from the Italian archive that were excluded in the published series because they were metastatic at diagnosis. These patients were radically resected on primary tumor but not on metastatic disease. The diagnosis of ACC was based on the pathological Weiss score [18]. Variables recorded included age, sex, hormone secretion, ENSAT stage at diagnosis [19], initial therapeutic options including primary surgery, disease-free survival (DFS) for patients initially radically resected, defined by the time elapsing from diagnosis to either disease relapse or patient death, overall survival (OS), calculated from diagnosis till death, Weiss score, mitotic count, sites of metastases at the time of progression. ACC relapse was defined as the appearance of local recurrence or metastatic disease at imaging techniques during follow-up. Adjuvant mitotane was offered to patients considered at high risk of relapse, in presence of the following criteria: 1) stage

III ACC; 2) high mitotic index. When a post-operative adjunctive measure was deemed necessary, a monitored mitotane treatment aiming at plasma concentrations between 14 and 20 mg/l [20,21] was employed. In the absence of intolerability to mitotane, treatment was scheduled for at least 2 years, or till ACC recurrence. Follow-up protocols were similar among the different centers including imaging (CT or MRI) of both chest and abdomen at baseline and thereafter every 3-6 months until disease progression or end of the study period. At each visit, the patients underwent physical examination, routine laboratory evaluation and hormonal work-up. Monitoring of mitotane concentrations was done in treated patients. For recurrent disease, radical surgery was performed if complete resection seemed feasible. In case of not resectable disease, patients received mitotane alone or chemotherapy plus mitotane according to disease aggressiveness, tumor bulk and previous mitotane exposure. Twenty-six patients, 4 metastatic at diagnosis and 22 with disease recurrence after primary surgery, received the combination of EDP-M. Patients gave informed consent for collecting tissue and clinical data and the study was approved by the ethics committees of both centres.

*RNA isolation from paraffin embedded tissues and quantitative real time PCR.*

Representative tumour areas were dissected under stereomicroscopic assistance from 10 µm sections of paraffin-embedded tissue in RNase-free conditions. RNA isolation was performed by commercially available paraffin material RNA extraction kits according to manufacturer's instructions (High Pure RNA Paraffin Kit; Roche Applied Science, Milano, Italy). Complementary DNA was transcribed using 500µg/ml oligodT (Roche Applied Science, Penzberg, Germany) and 500M-MLV RT (200U/µl) (Invitrogen, Carlsbad, California) according to standard protocols. Relative cDNA quantification of TS and TOP2A and of internal reference gene (beta-actin) was done in duplicate using a fluorescence-based real-time detection method (ABI PRISM 7900 Sequence Detection System-Taqman; Applied Biosystems, Life Technologies). Primers and probes sequences for TS and cycling conditions have already been published elsewhere [21]. TOP2A was

analyzed using the Mm00495701\_g1 assay (Applied Biosystems, Life Technologies). Baseline and threshold for cycle (Ct) calculation were set manually with ABI Prism SDS 2.1 Software. A mixture containing Human Total RNA (Stratagene, La Jolla, CA) was used as control calibrator on each plate.

The fold change in gene expression levels, expressed in unitless values, was evaluated using the  $2^{-\Delta\Delta C_t}$  method.

### *Statistical analyses*

Correlation between the expressions of the 2 genes was tested using the Spearman coefficient, differences of categorical variables were analyzed using the chi2 test. DFS and OS survival curves were computed using the Kaplan–Meier method and compared using the log-rank test. Hazard ratios (HR) for disease progression and patient death were estimated using the Cox proportional hazard model. Multivariate analyses were carried out adjusting for patient age, sex, Weiss score, ACC stage, mitotic count, and cortisol secretion. Cox models were also used to assess the presence of heterogeneity in the effect of marker expression in the different patient subgroups, defined by the covariates, by including in the model the appropriate treatment/covariate interaction term. Statistical analyses were carried out by using the SPSS for windows software (version 17).

## **Results**

### *Patients*

Patient characteristics are depicted in Table 1. Both Italian and German cohorts were comparable in terms of age, sex proportion, presence of clinical syndromes, and tumor characteristics (data for 92 patients previously published, [13]). Adjuvant mitotane therapy was administered to 38 patients (38.8%): 18 Italian and 20 German patients, respectively; the remaining 54 patients (55.1%) with non metastatic disease did not receive any postoperative treatment. The median follow-up was 66.3 months in all patients, being 80 months and 62.8 months in the Italian and German cohorts,



respectively. Among patients with stage II-III disease, 64 (65.3%) developed disease recurrence, 29 of the Italian series (67.4%) and 35 of the German series (74.5%). Among them, 30 were treated with chemotherapy; streptozotocyn in 8 patients, EDP (Etoposide, Doxorubicin, Cisplatin) in 22 patients. Both streptozotocyn and EDP were administered in association with mitotane. Overall 51 patients (52.0%) died of ACC progression: 28 (54.9%) in the Italian series and 23 (48.9%) in the German series, respectively.

#### *Relationship between tissue marker expression and patient and tumor characteristics*

As outlined in Table 2, both TOP2A and TS gene expression, dichotomized at the median value, did not show any significant relationship with histopathologic features, except for the significant direct correlation between TS expression levels and Weiss score. TOP2A and TS expressions were significantly directly correlated ( $p=0.006$ ) and both markers showed a direct relationship with RRM1 ( $p=0.026$  and  $p=0.011$  for TOP2A and TS, respectively) but not with ERCC1 ( $p=0.54$  and  $p=0.61$  for TOP2A and TS, respectively).

#### *Relationship between marker expression and disease free and overall survival*

In univariate analysis, TS gene expression was not associated with DFS [Hazard Ratio (HR): 0.96, 95% Confidence Interval (CI): 0.60-1.53,  $p=0.87$ ] and OS (0.86, 95% CI: 0.49-1.50,  $p=0.60$ ). High TOP2A expression levels were associated with higher risk of disease recurrence but just failing to attain statistical significance (HR: 1.49, 95% CI: 0.93-2.37,  $p=0.09$ ) and was significantly associated with a higher risk of death (HR: 1.78, 95% CI: 1.02-3.19,  $p<0.05$ ). In multivariate analysis, however, TOP2A expression failed to be a significant independent prognostic parameter either in terms of DFS (HR: 1.18, 95% CI: 0.71-1.98,  $p=0.51$ ) or OS (HR: 1.61, 95% CI: 0.87-2.98,  $p=0.13$ ).

### *Predictive role of TOP2A and TS expression for the efficacy of adjuvant mitotane*

As previously reported, in this series mitotane-treated patients had a longer median DFS than untreated patients (22.5 months [95% CI, 1.8–43.1] versus 13.2 months [95% CI, 6.2–20.2], HR, 0.70 [95% CI, 0.43–1.16; P= 0.17]), and longer median OS (154 months [95%CI, 65.1–242.9] versus 53 months [95% CI, 22.6–83.4], HR 0.63 [95% CI, 0.34–1.16; P=0.14]).

The prognostic effect of mitotane administration was assessed stratifying the patients according to marker expression (dichotomised at the median value). As shown in Figure 1, no different effect of mitotane treatment was observed in terms of DFS in patients with high versus low TOP2A and TS expression.

### *Predictive role of marker expression of EDP + mitotane activity*

Among the 26 ACC patients who received first line EDP-M for metastatic disease, four (15%) achieved a partial response (PR), nine (35%) stable disease (SD) and 13 (50%) experienced progressive disease (PD) after 2 or 3 cycles.

The clinical benefit (SD or PR) of EDP-M was directly associated with TOP2A expression: 12 SD/PR out of 17 (71%) patients in high TOP2A group as opposed to 1/9 (11%) in low TOP2A group (p=0.0039) (Figure 2a). Stratifying patients according to TS expression, a SD/PR was observed in 9/13 (69%) patients with high gene expression and 4/13 (31%) patients with low TS expression, respectively (p=0.049) (Figure 2b). EDP-M administration was associated with longer time to progression (TTP) in patients with high TOP2A as opposed to those with low TOP2A (p=0.038) (Figure 3), while no difference in terms of TTP after EDP-M was observed stratifying patients according to TS (data not shown).

Neither ERCC1 nor RRM1 correlated with a clinical benefit of the therapy: SD/PR in 8/15 (53%) versus 5/11 (45%) in high and low ERCC1 expressing tumors (p=0.69) (Figure 2c); SD/PR in 7/16 (44%) versus 6/10 (60%) in high and low RRM1 expressing tumors, respectively (p=0.42) (Figure

2d). No difference in term of TTP was observed stratifying patients according to both RRM1 and ERCC1 expression (data not shown).

## **Discussion**

TOP2A and TS have been shown to play a prognostic role in several cancers, such as colorectal, esophageal, breast, prostate, pancreatic, lung, and other cancers [23-37]. At the best of our knowledge, the prognostic role of these two markers has never been explored in ACC.

In this study, we have measured the mRNA expressions of TOP2A and TS in a relatively large series of 98 ACC patients, considering the extreme rarity of this tumor.

The results showed that TS expression is not prognostic in ACC while TOP2A expression in univariate analysis showed a significant correlation with a worse OS although failing to be significantly associated with DFS. In multivariate analysis, after adjusting for prognostic factors including mitotic index, TOP2A expression failed to be a significant independent prognostic parameter. TOP2A is a marker of cell proliferation [38] and the direct correlation of this enzyme expression with the mitotic count (although not significant) could account for its failure to be an independent prognostic parameter.

As mentioned in the introduction, both TOP2A and TS are also well known predictors of chemotherapy efficacy. In our series, high TOP2A expression in primary ACC was significantly associated with responsiveness to EDP-M and increased TTP in metastatic patients. TOP2A is a notorious predictor of efficacy to anthracycline and topoisomerase inhibitors in several tumors, but not to cisplatin. However, cisplatin is the reference cytotoxic drug in ACC and it is actually unknown whether EDP is more efficacious than cisplatin alone since no randomized studies have been conducted. In our opinion, these data suggest the importance of adriamycin and etoposide in the EDP scheme, particularly if TOP2A is highly expressed.

Also TS showed a direct relationship with the disease response of EDP-M, although with a lesser magnitude than TOP2A, but not with TTP. Since fluoropyrimidines are not included in the EDP-M

regimen, the direct relationship of TS with TOP2A may explain the association of this marker expression with EDP-M activity.

In this small series, both RRM1 and ERCC1 failed to be associated with EDP-M activity, this is an expected finding for RRM1 since this marker was repeatedly found to be predictive of gemcitabine efficacy, but not for ERCC1 that is a predictive factor of cisplatin efficacy in non small cell lung cancer [39]. In ACC patients, tissue ERCC1 assessed by immunohistochemistry was associated with efficacy of cisplatin containing regimens in one study [40] but not confirmed in another series [41].

In this paper we also explored the potential role of TOP2A and TS for mitotane efficacy in adjuvant setting. Although very recently sterol-O-acyl-transferase 1 (SOAT1) was identified as a key intracellular target of mitotane (41), the identification of a possible association between mitotane efficacy with a biological parameter in an explorative analysis, would be very useful to understand the mechanisms of sensitivity and resistance of ACC to this drug.

Both TOP2A and TS failed to have a predictive role for adjuvant mitotane efficacy; however the difference in the DFS curves of adjuvant mitotane versus follow-up only in low versus high TS expressing ACC, although not significant, does not exclude the potential role of this marker of mitotane efficacy.

In conclusion, the predictive role of TOP2A expression with the efficacy of EDP-M is new and of potential clinical relevance. EDP is a toxic regimen and this observation, if confirmed, suggests the potential importance of adding adriamycin and etoposide to cisplatin in highly expressing TOP2A tumors, while these 2 drugs might be unhelpful in low TOP2A expressing ACC.

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